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INVITED REVIEW

Interaction of reactive oxygen species with ion transport mechanisms

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ABSTRACT

The use of electrophysiological and molecular biology techniques has shed light on reactive oxygen species (ROS)-induced impairment of surface and internal membranes that control cellular signaling. These deleterious effects of ROS are due to their interaction with various ion transport proteins underlying the transmembrane signal transduction, namely, *1*) ion channels, such as Ca^{2+} channels (including voltage-sensitive L-type Ca^{2+} currents, dihydropyridine receptor voltage sensors, ryanodine receptor Ca^{2+}-release channels, and D-*myo*-inositol 1,4,5-trisphosphate receptor Ca^{2+}-release channels), K^+ channels (such as Ca^{2+}-activated K^+ channels, inward and outward K^+ currents, and ATP-sensitive K^+ channels), Na^+ channels, and Cl^- channels; *2*) ion pumps, such as sarcoplasmic reticulum and sarcolemmal Ca^{2+} pumps, Na^+-K^+-ATPase (Na^+ pump), and H^+-ATPase (H^+ pump); *3*) ion exchangers such as the Na^+/Ca^{2+} exchanger and Na^+/H^+ exchanger; and *4*) ion cotransporters such as K^+-Cl^-, Na^+-K^+-Cl^-, and P_i-Na^+ cotransporters. The mechanism of ROS-induced modifications in ion transport pathways involves *1*) oxidation of sulfhydryl groups located on the ion transport proteins, *2*) peroxidation of membrane phospholipids, and *3*) inhibition of membrane-bound regulatory enzymes and modification of the oxidative phosphorylation and ATP levels. Alterations in the ion transport mechanisms lead to changes in a second messenger system, primarily Ca^{2+} homeostasis, which further augment the abnormal electrical activity and distortion of signal transduction, causing cell dysfunction, which underlies pathological conditions.

ischemia-reperfusion; muscle pathologies; thiol group; calcium homeostasis; membrane compartmentation; reducing and oxidizing agents

INTRODUCTION

▲ Top
▲ Abstract
• Introduction
▼ Conclusions
▼ References

REACTIVE OXYGEN SPECIES (ROS), such as superoxide radical anion (O^-_2), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical (· OH), and hypochlorous acid (HOCl), are produced as by-products of oxidative metabolism, in which energy activation and electron reduction are involved. Their production is enhanced during inflammation, aging, radiation exposure, endotoxic shock, and ischemia-reperfusion of heart, intestine, liver, kidney, and brain. They have been implicated in various cell dysfunctions (91, 126). This can be indicated by the protection provided following treatments with free radical-scavenging enzymes (9, 54, 94, 104). The mechanisms of ROS action at the cellular level are not well understood. It is obvious, therefore, that the understanding of these mechanisms is important for developing therapeutic strategies at cellular sites of dysfunction. In particular, the role of cell membranes in compartmentation and transmembrane signal transduction renders the changes in their properties the early events that are associated with cell dysfunction. This review examines the interaction of ROS with membrane phospholipids and proteins that constitute ion transport pathways, i.e., ion channels, pumps, exchangers, and cotransporters of both internal and surface membranes in general and in muscles in particular.

PRODUCTION, IDENTIFICATION, AND PATHOLOGIES OF ROS

The metabolic pathways that are known to produce ROS include *1*) the xanthine (X)/xanthine oxidase (XO) system, *2*) the cyclooxygenase pathway of the arachidonic acid metabolic system, *3*) the electron transport system of mitochondria, *4*) the activated neutrophil system, and *5*) the amyloid β protein system. The significance of the contribution of each of these ROS sources is not well understood.

The superoxide radical anion O^-_2 is produced by the reduction of O_2 using an electron that can be supplied by superoxide-generating NADPH oxidase as follows

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+ \quad (1)$$

In aqueous solution, the production of H_2O_2 is as follows

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + Fe^{2+} + O_2 \quad (2)$$

(dismutation reaction)

The Fe^{3+}-induced catalysis of · OH production is shown in the Fenton reaction (61) as follows

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \quad (4)$$

(Fenton reaction)

$$\text{Net}: O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH \quad (5)$$

Experimentally, different ROS-generating and/or ROS-identifying systems have been used to examine the ROS-induced modifications of ion transport pathways (see Tables 1-9). These include *1*) H_2O_2; *2*) *tert*-butyl hydroperoxide (*t*-BHP), a substrate of glutathione peroxidase; *3*) *t*-butoxy (RO ·) or *t*-butylperoxy (ROO ·) radical-generating systems; *4*) hypoxanthine (HX)/XO, a source for O^-_2, H_2O_2, and · OH production; *5*) dihydroxyfumaric acid (DHF); *6*) cumene hydroperoxide or purine/XO; *7*) photooxidizing rose bengal, a source for $^1O_2$; *8*) ionizing γ-irradiation, diethylenetriaminepentaacetic acid, and catalase/XO; *9*) HX/XO, $FeCl_3$, and ADP; *10*) phorbol myristate acetate activation of H_2O_2 production in neutrophils; and *11*) the free radical scavenger *N*-acetyl-L-cysteine. Pharmacological identification and dissection of the combined ROS effects are achieved by examining the modulatory effects of specific scavengers for H_2O_2, O^-_2, · OH, and $^1O_2$ such as catalase, superoxide dismutase (SOD), desferrioxamine, and histidine, respectively. It is thought that the effects of the non-free-radical H_2O_2 are caused by producing more highly reactive oxygen species such as the free radicals O^-_2 and · OH. In particular, · OH reacts rapidly with many substances, e.g., DNA, lipid, and carbohydrates.

A flowchart of the major processes of ROS underlying pathologies is shown in Fig. 1. The pathologies that have been attributed to ROS-induced cell dysfunction include *1*) cardiac stunning and arrhythmia (see Refs. 35 and 61); *2*) skeletal muscle injury (see Refs. 130 and 151); *3*) neurological conditions (see Refs. 91 and 126), e.g., neuronal damage in Parkinson's disease (see Ref. 27); *4*) neurotoxicity (107); *5*) Alzheimer's disease (see Refs. 6 and 171); *6*) diabetes (see Ref. 123), apoptosis of T lymphocytes (see Ref. 37), and gastric mucosal injury (see Ref. 160); and *7*) hypertension (156). Some of these effects can be suppressed by free radical scavengers (9, 54, 94, 104).

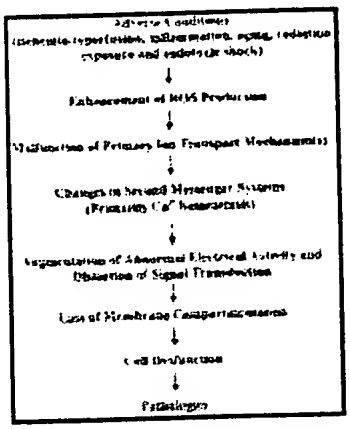

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Fig. 1. Flow chart of major processes of reactive oxygen species (ROS) pathologies.

In skeletal muscle, exercise increases the rate of ROS production (30, 130, 158). The enhancement of ROS production due to the increase in activity of mitochondrial electron carriers, low catalase concentrations, the sudden changes in oxygen supply and consumption, and the presence of high levels of myoglobin acting as a catalyst for the formation of oxidants is thought to cause skeletal muscle injury (see Ref. 130). The increase of free radicals in skeletal muscle and liver cells during exhaustive exercise is associated with a decrease in mitochondrial respiratory control, loss of sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) integrity, and increased levels of peroxidation products and lipid peroxidation. These effects are similar to those observed in vitamin E-deficient animals (30).

ROS INTERACTION WITH ION TRANSPORT PATHWAYS

The interaction of ROS with ion transport pathways in muscles can be deduced indirectly from changes in their membrane properties. Cosentino et al. (29) demonstrated the role of O^{-}_{2} in the mediation of endothelium-dependent contraction. It has also been demonstrated that H_2O_2 potentiates twitch tension in cardiac (84, 136) and skeletal muscles (124, 136), and this induced tension can be decreased by catalase, a specific enzyme that hydrates H_2O_2 (136). The effects are usually characterized by amplification of tension and tension oscillation, followed by spontaneous contractions (84, 124). This effect of H_2O_2 is not mediated via end effects on the myofilaments (111, 124). This suggests that the signal transduction pathways are affected by ROS. Early studies revealed that the effects of ROS on membrane properties could be deduced from electrophysiological parameters of the membrane. These include changes in membrane current and potential, ionic gradients, action potential duration and amplitude, afterdepolarization, and spontaneous activity and loss of excitability (see Refs. 40, 166, 167).

The effects of ROS-generating systems on membrane potential are now well established. It has been

demonstrated that X/XO as a ROS-generating system caused membrane depolarization and a decrease in the action potential amplitude and maximum rate of rise of action potentials in guinea pig ventricular myocardium (127). Delayed afterdepolarization and early afterdepolarization induced by *t*-BHP, DHF, and X/XO in guinea pig papillary muscle and canine ventricular myocytes have also been demonstrated (4, 5, 122). ROS-induced membrane depolarization has been attributed to inhibition of a Na^+ current (11) or an inward K^+ current (121), activation of an inwardly directed nonselective cation current (115, 152), and increase in a Ca^{2+} current that is associated with changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Similarly, the oscillation in $[Ca^{2+}]_i$ has been implicated in arrhythmogenic afterdepolarization (113).

ROS-induced shortening of the action potential duration has been attributed to a possible increase in a delayed rectifying K^+ current and decrease in activation of ATP-sensitive K^+ (K_{ATP}) channels and Ca^{2+} currents (121, 146). Exogenous ROS-induced changes in the electromechanical function and metabolism in isolated rabbit and guinea pig ventricles shortened the duration of the action potential, indicating a decrease in the Ca^{2+} current and time-dependent outward current (50). More recently, Tokube et al. (169) reported biphasic changes in the action potential duration, with initial lengthening of the action potential due to a rapid decrease in whole cell K^+ currents and subsequent shortening due to a decrease of whole cell Ca^{2+} current and increase in the single ATP-sensitive time-dependent outward K^+ current.

In cardiac, smooth, and skeletal muscles the deleterious effects of ROS, produced by leaked electrons from the electron transport system of the mitochondria, are due to their interaction with various ion transport proteins underlying transmembrane signal transduction (Fig. 2). Figure 2 indicates that an important feature of ROS interaction with ion transport proteins is the modification in Ca^{2+} homeostasis that ultimately causes muscle pathologies.

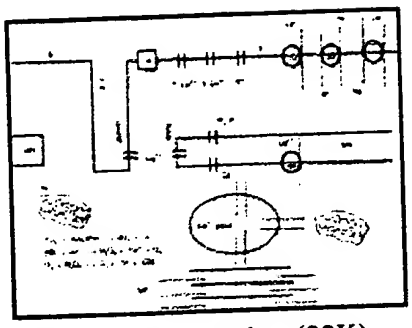

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Fig. 2. Generalized scheme showing ROS-modulated ion transport mechanisms that control Ca^{2+} homeostasis (Ca^{2+} pool) in muscles. These transport mechanisms include *1*) ion channels: Ca^{2+} channels, including voltage-sensitive L-type Ca^{2+} currents (V Ca^{2+}), ligand Ca^{2+} channels (R Ca^{2+}), dihydropyridine receptor (DHPR) voltage sensor, ryanodine receptor (RyR) Ca^{2+}-release channels, and D-*myo*-inositol 1,4,5-trisphosphate receptor (IP_3-R) Ca^{2+}-release channels; K^+ channels, such as ATP-sensitive K^+ channels; and Cl^- channels, such as the small Cl^- (SCl) channel, *2*) ion pumps: such as sarcoplasmic reticulum (SR) and sarcolemmal (S) ATP Ca^{2+} pumps and Na^+-K^+-ATPase (Na^+ pump); and *3*) ion exchangers: Na^+/Ca^{2+} exchanger. Excited cell membrane (sarcolemma of skeletal muscle) or specific receptor (R; in sarcolemma of cardiac and smooth muscles) communicate with the Ca^{2+} sender (SR in skeletal and smooth muscles and sarcolemma in cardiac muscle) by means of the T tubule (T-T) or by second messengers, such as cAMP (cardiac muscle) or IP_3 (smooth muscle). M, mitochondria; MF, myofilaments; R, receptor β (cardiac muscle) and α_1 (smooth muscles).

Ion Channels

Ca^{2+} channels. L-TYPE VOLTAGE-SENSITIVE CA^{2+} CURRENTS. L-type voltage-sensitive Ca^{2+} channels play an important role in Ca^{2+} homeostasis in ventricular myocytes. Hence numerous studies have been conducted to examine the effects of ROS on these channels and to determine their contribution to the alterations in Ca^{2+} homeostasis under adverse conditions (Table 1). It appears that the data for the effects of ROS on current peak, amount of current, and kinetics of L-type Ca^{2+} channels in ventricular myocytes are conflicting. It has also been reported that H_2O_2 has no influence on L-type Ca^{2+} current (100, 101). In contrast to the finding that ROS-induced reduction in peak current was associated with an increase in mean current due to slowing of the inactivation (26), Tokube et al. (169) reported a decrease in the current peak with no changes in the activation time course of this current. On the other hand, Cerbai et al. (20), Matsuura and Shattock (115), and Moghadam and Winlow (119) reported a decrease in L-type Ca^{2+} current. This decrease has been attributed to Ca^{2+}-induced channel inactivation (see Ref. 115). The H_2O_2-induced decrease in the inward Ca^{2+} current in cultured *Lymnaea* neurons is dose dependent (119). There are data suggesting that overload due to Ca^{2+} influx through the voltage-gated Ca^{2+} channel can be ruled out, since free radicals and H_2O_2 inhibit the voltage-sensitive L-type Ca^{2+} current (48, 50, 51, 121). The inhibitory effects of HX/XO and DHF as ROS-generating systems were reversed with SOD and catalase, suggesting that both O^-_2 and H_2O_2 are effective (Table 1), whereas the effects of the

cumene/XO ROS-generating system were irreversible (48). Internal oxidative agents used on ion channels also show that 4,4'-dithiodipyridine [DTDP; a lipophilic sulfhydryl (SH)-oxidizing agent] and thimerosal {[(*o*-carboxyphenyl)thio]ethyl mercury sodium salt, a hydrophilic SH-oxidizing agent} inhibit the activity of cloned rabbit smooth muscle L-type Ca^{2+} channels (23).

Table 1. Effects of ROS on DHPR and L-type Ca^{2+} currents

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Ca^{2+} channel blockers have been used to identify the Ca^{2+} pathway contributing to changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). The Ca^{2+} channel blocker nifedipine blocks O^-_2-induced increases in $[Ca^{2+}]_{cyt}$ in human myometrial cells (112). Although indirect and inconclusive, the finding is taken by the authors to indicate that the increase in Ca^{2+} is mediated via an O^-_2-affected voltage-sensitive L-type Ca^{2+} channel. Recently, Ueda et al. (171) reported that free radicals, monitored with 2',7'-dichlorofluorescin diacetate, may be involved in amyloid protein potentiation of Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels. Amyloid β protein, which accumulates in the brain of Alzheimer patients, generates Ca^{2+}-independent free radicals that potentiate the influx of Ca^{2+} through L-type voltage-sensitive Ca^{2+} channels in rat cultured cortical and hippocampal neurons. The neurotoxicity (see Refs. 6 and 170) caused by this influx is attenuated by nimodipine (171, 180) and vitamin E (171).

DIHYDROPYRIDINE RECEPTOR VOLTAGE SENSOR. There is indirect evidence for the effect of ROS on the dihydropyridine receptor (DHPR). It has been found that H_2O_2 prevents Ag^+ contractions and Ag^+ inhibition of excitation-contraction (E-C) coupling in single skeletal muscle fibers from *Rana temporaria* or *R. catesbeiana* (124). Recently, Oba et al. (124) proposed that H_2O_2 induces skeletal muscle dysfunction by acting on the DHPR and the ryanodine receptor (RyR) in T tubule and SR, respectively. Tension experiments on skinned single muscle fibers from *R. catesbiana* reveal that 1.5-6 mM H_2O_2 potentiates decaying twitches indicative of a direct action on the DHPR, although neither resting nor action potentials were affected (124). Decaying twitches were seen in the presence of 5 mM dithiothreitol (DTT) and were amplified and slowed with BAY K 8644. Binding studies that also indicate a ROS-induced decrease in this current may suggest a direct effect on the channel protein. Kaneko et al. (87) observed a reduction in DHP binding sites in the membranes of heart cells exposed to oxygen free radicals. Similarly, in guinea pig ventricular myocytes, the ROS-generating system DHF reduced [^{3}H]PN-200-110 binding sites of DHP, underlay the observed reduction in L-type Ca^{2+} currents, and was prevented by SOD and catalase (58). These authors postulated that these changes in the DHPRs, which reduce Ca^{2+} currents, mediate the mechanical dysfunction associated with oxidative stress. However, the effects of H_2O_2 or other ROS on single DHPR channel activity have not been reported yet.

RYR CA^{2+}-RELEASE CHANNELS. In cardiac and skeletal muscles the RyR Ca^{2+}-release channels are essential in maintaining Ca^{2+} homeostasis that underlies the mechanism of muscle contraction and relaxation. There are only a few studies regarding the effects of ROS on these channels. However, these studies have established that the effect of ROS on Ca^{2+} homeostasis can be attributed, in part, to Ca^{2+} release from the SR. There is also biochemical evidence revealing that ROS modify the structure and function of the cardiac SR RyR Ca^{2+}-release channel, where the initial increase in the probability of the channel being in the open state (P_o) is followed by irreversible loss of the channel function (69). In skeletal muscle, H_2O_2 induces SR Ca^{2+} release that can be enhanced with Cu^{2+} (170), probably through skeletal SR RyR Ca^{2+}-release channels (124). Similarly, in sheep cardiac SR, H_2O_2 (3-5 mM) directly modified the gating of the RyR Ca^{2+}-release channel, causing an increase in the P_o, without affecting the conductance or channel modulation with ATP, caffeine, Mg^{2+}, or ryanodine (12). It appears that Ca^{2+} release from the SR can be induced using different ROS-generating systems (Table 2). For example, a 106-kDa Ca^{2+}-release channel protein from the SR of skeletal muscle is also activated by rose bengal (185). More recently, using the Ca^{2+} sensitivity and the maximum Ca^{2+}-activated force of isolated skinned fibers as indirect investigative parameters, Posterino and Lamb (132) reported that reducing agents do not inhibit the E-C coupling and that oxidizing agents do not cause a significant Ca^{2+} release under physiological conditions. However, in the absence of supporting data at the single Ca^{2+} channel and ion pump levels, it is difficult to determine any direct modifications in the Ca^{2+}-transport pathways or other mechanisms of Ca^{2+} release and uptake.

Table 2. Effects of ROS on RyR Ca^{2+}-release channels

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D-*MYO*-INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR CA^{2+}-RELEASE CHANNELS. ROS-induced Ca^{2+} release via modifications in D-*myo*-inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release at the single-channel level remains to be experimentally observed. However, it has been reported that O^-_2 stimulates IP_3-induced Ca^{2+} release from the SR of vascular smooth muscle (165). Furthermore, it has been proposed that SH reagents may induce Ca^{2+} release by sensitizing the IP_3 Ca^{2+}-release receptor (118). The data reported by Elmoselhi et al. (43) indicate that oxygen free radicals modify IP_3-sensitive Ca^{2+} channels (see *Changes in Ca^{2+} Homeostasis*). It has also been reported that oxidized glutathione (GSSH) decreases the luminal Ca^{2+} content of the endothelial cell line IP_3-sensitive Ca^{2+} store (64).

K^+ channels. CA^{2+}-ACTIVATED K^+ CHANNELS. The role of ROS in modulating ion channels has also been inferred from the use of ion channel blockers together with ROS-generating systems. The K^+ channel blocker quinidine hydrochloride reduced Ca^{2+}-dependent chemiluminescence products, indicative of oxygen radical production, in human eosinophils (143). They postulated that production of oxygen free

radicals by the membrane-bound NADPH oxidase may be mediated by Ca^{2+}-activated K^+ (K_{Ca}) channels in human eosinophils and that this mechanism may underlie the role of eosinophils in the pathogenesis of allergic diseases. Relaxation evoked by nonneurogenic electrical field stimulation, via generation of free radicals, also modified Ca^{2+}-dependent channels (1, 81, 186). In contrast to the H_2O_2-induced reversible inhibition [with DTT and reduced glutathione (GSH)] of K_{Ca} channels in the plasma membrane of bovine aortic endothelial cells (18), the large K_{Ca} channel in skeletal muscle from mouse is insensitive to as high as 50 mM H_2O_2 concentration (179). Differences in H_2O_2-induced modification in channel activity could be attributed to difference in tissue types. For example, it has been found that reducing agents decrease the activity of K_{Ca} channels in pulmonary, but not in ear, arterial smooth muscle cells of rabbit (128). The significance of H_2O_2 inhibition of K_{Ca} channels derives from the fact that disruption of Ca^{2+} homeostasis is mediated via depolarization of the membrane potential (see Table 3) (18).

Table 3. Effects of ROS on K_{Ca} channels

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INWARD AND OUTWARD K^+ CURRENTS. The modifications in ion channels that underlie ROS-induced changes in the duration of action potential in cardiac cells include, in addition to the ROS-induced decrease in L-type Ca^{2+} current, a suppression of the delayed outward and the inward K^+ currents (77, 169). Electrically evoked relaxation, which generates free radicals in canine airway smooth muscle relaxation, was not sensitive to removal of external K^+ but was sensitive to tetraethylammonium, high KCl concentrations, charybdotoxin, quinine, and free radicals (186). This was deduced from the action of the free radical scavenger *N*-acetylcysteine and was mimicked by H_2O_2, whereas SOD and catalase were ineffective. In guinea pig ventricular myocytes the ROS-generating system DHF also reduced the outward K^+ current that determines the prolongation of the action potential as a result of exposure to oxygen free radicals (20, 58). Similarly, Kuo et al. (104) found that nonlethal ionizing γ-irradiation (10 cGy) transiently ($t_{1/2}$ 90 min) induced whole cell voltage-dependent outward K^+ currents, mimicking H_2O_2 and heat stress in activating this current, with no changes in membrane potential of −70 mV, intracellular K^+ concentration ($[K^+]_i$), or ATP levels.

Several inward and outward K^+ channels are affected by different ROS-generating systems (Table 4). In guinea pig cardiac ventricular myocytes HX/XO (ROS production that is indicated from adrenochrome formation from adrenaline) decreased the inward K^+ current (26). In rabbit sinoatrial atrioventricular node preparation, *t*-BHP transiently increased the spontaneous firing frequency, increased the amplitude of the action potential, and induced biphasic changes in Ca^{2+} current, delayed rectifying K^+ current, and hyperpolarization-activated inward current (145). In guinea pig ventricular cells cumene hydroperoxide decreased whole cell inward rectifier K^+ current and inhibited the single inward rectifier K^+ channel

without altering the unit amplitude of single-channel current (121). In *Xenopus* oocytes H_2O_2 reversibly and specifically inhibited the time-dependent fast activation of a certain voltage-gated K^+ channel concomitantly associated with an increase in K^+ currents of cloned K^+ channels KShIIIC, KShIIID, and HukII. Other cloned voltage-dependent channels were not affected by 1.6 mM H_2O_2, e.g., *Shaker* 29-4 and KShIIIA.1 (173). Recently, Dupart et al. (37) found that photoactivation of rose bengal induced inhibition of the cloned K^+ channel activity of *Shaker* channels Kv1.3, Kv1.4, and Kv1.5, *Shaw* channel Kv3.4, and inward K^+ rectifier IRK3, whereas *Shaker* Kv1.2, *Shab* channels Kv2.1 and Kv2.2, *Shal* channel Kv4.1, and inward rectifiers IRK1, ROMK1, and hIsK were not affected. On the other hand, *t*-BHP removed the fast inactivation of the *Shaker* K^+ channels Kv1.4 and Kv3.4, whereas other K^+ channels were not affected. These findings indicate that ROS effects depend on the ROS-generating system as well as on the type of channel protein under investigation.

Table 4. Effects of ROS on inward, outward and other K^+ currents

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ATP-SENSITIVE K^+ CHANNELS. The effects of H_2O_2 and HX/XO on K_{ATP} channels in cardiac and pancreatic cells have been reported (Table 5). Their effects on this channel are reminiscent of the effects of hypoxic conditions. For example, hypoxic conditions induced a time-independent K^+ current through K_{ATP} in isolated heart cells of the guinea pig (7). Similarly, in guinea pig ventricular myocyte cells, the HX/XO ROS-generating system increased the P_o of K_{ATP} and glibenclamide-sensitive K^+ channels (77, 169). Concentrations of >30 μM H_2O_2 also increased the activity of K_{ATP} channels in the plasma membrane of rat pancreatic β-cells in whole cell current of perforated cells but not that of conventional whole cell configuration. Furthermore, it increased single-channel activity in the cell-attached configuration but not in the inside-out configuration. This effect was inhibited with tolbutamide, glyceraldehyde, and 2-ketoisocaproic acid (123). Evidence for direct effects of H_2O_2 on K_{ATP} channels can be deduced from studies where ROS effects were examined on excised membrane patches. For example, Ichinari et al. (73) observed a dose-dependent H_2O_2-induced increase in P_o of the K_{ATP} channel in the isolated inside-out configuration. It has been proposed that a H_2O_2-induced increase in K_{ATP} channel activity in β cells that remained sensitive to ATP was due to indirect channel opening via inhibition of glycolysis and/or oxidative phosphorylation, leading to a decrease in the cytosolic concentration of ATP (123), as previously suggested for the H_2O_2-induced increase in K_{ATP} channels in guinea pig ventricular myocytes (50). In β cells, H_2O_2 stimulated K_{ATP} currents but not Ca^{2+} current (100, 101). They proposed that the SH-oxidizing agents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and H_2O_2 may act *1*) on different SH targets or *2*) via a mechanism other than oxidation of SH groups. H_2O_2-induced relaxation in rabbit airway smooth muscle has been attributed to the activation of

an α-catalytic subunit with a relative molecular weight (M_r) of 90,000-110,000 and a β-subunit with an M_r of 40,000-60,000. The Na^+ pump is important for maintaining coronary tone. The pump transports three Na^+ and two K^+ per one ATP hydrolyzed against their concentration gradients, generating internal negative charges. The intracellular/extracellular concentrations in muscle cells are typically 5-115/130 mM Na^+ and 130/5 mM K^+ (see Ref. 42). There are several reaction steps in the function of the pump. The hydrolytic and transport reactions of the pump can be uncoupled. For example, treatment of inside-out erythrocyte vesicles with trypsin or chymotrypsin uncouples the transport of Na^+ from the Na^+-K^+-ATPase (62). K^+-activated ouabain-sensitive *p*-nitrophenylposphatase is associated with the hydrolytic step of the Na^+ pump. Conflicting effects of ROS on Na^+ pumps have been reported (see Ref. 62). Some of these differences in ROS effects are due to the methods used for ROS generation, in which different individual ROS may not act via the same mechanism (Table 8). These differences could also arise from resolution limitations of the flux experiments, which can be overwhelmed by the back flux of ROS-enhanced activated K^+ channels.

Table 8. Effects of ROS on Na^+-K^+-ATPase (Na^+ pump)

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Kim and Akera (90) examined Na^+-K^+-ATPase or the Na^+ pump in the sarcolemma of guinea pigs. Na^+-K^+-ATPase activity was deduced from ouabain-sensitive ATPase and was estimated from ouabain-sensitive $^{86}Rb^+$ uptake. They found that scavengers (100 U/ml SOD, 150 U/ml catalase, 50 mM DMSO, 10 mM histidine, and 50 μg/ml vitamin E or 1 mM XO inhibitor allopurinol) of O_2 free radicals inhibited ischemia and reperfusion-induced reduction in Na^+-K^+-ATPase activity and specific [^{3}H]ouabain binding. The pump also appeared to be sensitive to changes in membrane phospholipids. It has been shown that Na^+-K^+-ATPase activity is also inhibited by lipooxygenase, dihydroxyfumarate, ascorbate-$FeCl_2$, and cumene hydroperoxide (15, 99, 163). Jamme et al. (80) reported that the inhibition of mouse cerebral Na^+-K^+-ATPase activity by ultraviolet C (UV-C)-generated · OH and peroxyl (ROO ·) radicals is mediated via lipid peroxidation-induced disruption of membrane integrity that consequently results in conformational changes, leading to inactivation of membrane-bound proteins. It has also been suggested that ascorbate-$FeCl_2$-induced inactivation of cerebral Na^+-K^+-ATPase is due to lipid peroxidation-induced reduction in the affinity for Na^+ and K^+ and an increase in ATP and ouabain affinities (117). The inhibitory effects of iron-generated free radicals on the activity of Na^+-K^+-ATPase can be reversed by antioxidants (138).

It appears that the effects of free oxide radicals during ischemia and/or perfusion on Na^+-K^+-ATPase are not due to depletion of ATP, since ATP recovers on reperfusion while the free oxide radicals continue to enhance the decrease in Na^+-K^+-ATPase and glycoside binding sites (see Refs. 82 and 90). The effects of X/XO on cardiac Na^+-K^+-ATPase are not clear. Vlessis et al. (175) reported inhibition of both Na^+

transport and the Na^+-K^+-ATPase, whereas Kukreja et al. (103) found that X/XO was ineffective while Na^+-K^+-ATPase was inactivated by H_2O_2, $HClO_4$, and NH_2Cl treatments. Vinnikova et al. (174) found that the $^1O_2$-induced inhibitory effect on Na^+-K^+-ATPase was prevented by the $^1O_2$ scavenger, histidine, whereas SOD, catalase, and mannitol were not effective in providing such protection. Therefore, these data rule out inhibitory effects due to O^-_2, H_2O_2, and · OH. Elmoselhi et al. (42) suggested that H_2O_2 and O^-_2 uncouple the hydrolytic activity of the Na^+ pump from $Rb^+(K^+)$ uptake. They proposed that such uncoupling under ischemic conditions and reperfusion would damage coronary artery smooth muscle as a result of continuous ionic imbalance and starvation of the cell via continuous ATP hydrolysis. The levels of H_2O_2 and O^-_2 required for uncoupling the Na^+ pump are higher than those affecting other processes; hence H_2O_2 and O^-_2 effects are unlikely to be directly due to uncoupling of the hydrolytic and transport reactions of the Na^+ pump. It is not known whether ROS affect the various Na^+ pump isoforms. For example, these isoforms differ in their α- and β-subunits and in their affinities to Na^+ and K^+ (42). Differences in responses of these isoforms to specific ROS may shed light on their molecular mechanisms of action at the subunit level. In this regard it has been found that the α_1 and α_2 isoforms of the Na^+-K^+-ATPase differ in their sensitivities to oxidants (71, 184).

H^+ pump. The H^+ pump is important for preventing a drastic intracellular acidification and for charge balance and membrane polarization. Oxidant stress-induced pH changes in peritoneal macrophages have been attributed to modifications in the plasmalemmal H^+-ATPase (see Ref. 14).

Adenine nucleotide translocator, phosphate carrier, and uncoupling proteins. These proteins are present in mitochondria. The phosphate carriers catalyze the electroneutral exchange of phosphate for hydroxyl ion. The adenine nucleotide carrier binds and transports adenine nucleotides, whereas uncoupling proteins bind purine nucleotides but transport H^+, OH^-, or Cl^- (see Ref. 137). The effects of ROS on these protein transporters are not known. However, it is very likely that they are affected by ROS. First, it is known that in skeletal muscle and liver cells free radicals increase during exhaustive exercise and this increase is associated with a decrease in mitochondrial respiratory control (30). Second, ROS have deleterious effects on mitochondrial metabolism (see Ref. 50) and are linked to a leakage of electrons from mitochondria (see Ref. 91). Third, the presence of the SH groups on the cysteine residues and the SH-induced modification in permeation of phosphate, Cl^-, and H^+ (137) also suggest that ROS may modify these transporters.

Ion Exchangers

Na^+/Ca^{2+} exchanger. The Na^+/Ca^{2+} exchanger couples the transport of three Na^+ to that of a single Ca^{2+} in the opposite direction in two consecutive, yet separate steps (see Ref. 28). The Na^+/Ca^{2+} exchanger, together with Ca^{2+}-ATPase of the ER/SR, regulates Ca^{2+} levels that underlie muscle contractility behavior under both normal and ischemic conditions (see Ref. 13). In cardiac muscle the Na^+/Ca^{2+} exchanger contributes to force development, in particular, under glycosidic conditions (see Ref.

131). In smooth muscle, relaxation is achieved partially via a decrease in $[Ca^{2+}]_{cyt}$ efflux at the plasmalemma by means of the Na^+/Ca^{2+} exchanger (159). There is evidence suggesting that this exchanger is a tetramer linked by disulfide bonds, and thus it is susceptible to modification by oxidizing and reducing agents as well as ROS (see Refs. 19, 88, 129). However, both decreases (24, 34, 88) and increases in Na^+-dependent Ca^{2+} uptake (Na^+/Ca^{2+} exchanger) (49, 135, 154) in both isolated and intact sarcolemmal vesicles have been reported (see Table 9). The exchanger is also inhibited by the oxidizing agent HOCl (46, 88), the SH-alkylating agent diamide (2, 24, 129), and SH-reducing agents GSH and DTT (131). There is also evidence that SH-alkylating diamide stimulates Ca^{2+}/Na^+ exchange (129). The nature of the inhibition or stimulation is not clear. The stimulation of the Na^+/Ca^{2+} exchanger has been attributed to the increase in affinity to Ca^{2+}, i.e., a decrease in K_m for Ca^{2+} (135, 154) with no changes in voltage dependency (154). The pathophysiological implication is that such stimulation of Na^+/Ca^{2+} exchange by ROS may moderate the myocardial response to ischemia-reperfusion injury (154). DiPolo and Beauge, (33) proposed that the inhibition is due to a reduction in the affinity of the exchanger to Ca^{2+}. The conflicting effects of ROS on the Na^+/Ca^{2+} exchanger may be partially due to the use of a different ROS-generating system and different parameters to deduce the exchanger activity (see Table 9). For example, Kato and Kako (88) found that HOCl induced inhibition whereas H_2O_2 induced stimulation of the Na^+/Ca^{2+} exchanger. H_2O_2-induced Cl^- current is used as an indicator of enhancement in the Na^+/Ca^{2+} exchanger (149). However, both Coetzee et al. (24) and Goldhaber (49) obtained conflicting data, despite the fact that both used Ni^{2+} sensitivity of a membrane current as a marker for the Na^+/Ca^{2+} exchanger. The conflicting effects of ROS on this exchanger are not due to the ROS-generating system, since it has been found that H_2O_2 and X/XO similarly enhanced this exchanger in ventricular myocytes, causing Ca^{2+} overload and triggering arrhythmia during reperfusion (49). The conflicting effects may be due to differences in the exchanger mode during which the effects of ROS were examined. There is evidence suggesting that Ca^{2+} and Na^+ are translocated in separate consecutive steps (see Ref. 89) and that the Na^+/Ca^{2+} exchanger may operate in reverse, i.e., efflux of Na^+ and influx of Ca^{2+} during ischemia-reperfusion when cytoplasmic concentration of Na^+ is increased (54). Furthermore, the exchanger is modulated by Ca^{2+} and/or ATP, which affect the exchange distribution between the active state and either of two inactive states (see Refs. 66 and 67). Other regulatory mechanisms may be involved, such as changes in lipid composition (106) and H_2O_2 production via insulin-NADPH oxidase interaction (88).

Table 9. Effects of ROS on Na^+/Ca^{2+} exchangers

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Na^+/H^+ exchange. The isoforms of the Na^+/H^+ exchanger are present in various epithelial and muscle cells. They play important physiological roles, such as regulation of intracellular pH, cell volume, and

reabsorption of NaCl and $NaHCO_3$. There is little information on the effects of ROS on these exchangers in epithelial cells. ROS have been implicated in the increased activity of the cellular Na^+/H^+ exchanger that is activated by phosphorylation in vascular myocytes from hypertensive rats (156). It has also been reported that exposure of human neutrophils to 100 nM *N*-formyl-methionyl-leucyl-phenylalanine activated the amiloride-sensitive Na^+/H^+ exchanger, leading to an increase in intracellular pH from 7.22 to 7.8 (157). The ROS-generating system X/XO inhibited this transport system in isolated myocytes of rat heart and in sealed sarcolemmal vesicles of bovine heart (184), and the inhibition was reversed with catalase and SOD and, therefore, indicated that H_2O_2 and O^-_2 were the effective moieties. The effect of ROS on the Cl^-/HCO^-_3 exchanger is unknown.

Ion Cotransporters

Cation-Cl^- transporters. The electroneutral transporters have important physiological roles, such as regulatory volume decrease and transepithelial salt transport (see Ref. 120). Their activity depends on the presence of all the transported ions. However, they differ pharmacologically with respect to the identity and stoichiometry of the transported ions. There is little direct or indirect information on the effect of ROS on these transporters.

K^+-CL^- COTRANSPORT. This cotransport system could also be modulated by ROS, since it has been reported that K^+-Cl^- cotransport in erythrocytes is modulated by SH groups. It is activated through *N*-ethylmaleimide (NEM)-induced SH alkylation and methylmethane thiosulfonate- or diamide-induced SH oxidation (see Ref. 187). It has also been found that phenazine methosulfate, a generator of oxygen free radicals, stimulated the reversible K^+-Cl^- cotransport system in human erythrocyte membranes (60).

NA^+-K^+-CL^- COTRANSPORT. The effects of oxidant stress, induced via cell incubation in *t*-BHP in the presence of bumetanide, show a decrease in the inward movement of Rb^+, indicating inhibition of the bumetanide-sensitive $^{86}Rb^+$ pathway, which represented Na^+-K^+-Cl^- cotransport (41). Similarly, *t*-BHP inhibited Na^+-K^+-Cl^- cotransport in skeletal muscle (151).

Other cotransporters. NA^+-P_I COTRANSPORT. H_2O_2 and O^-_2 inhibited the Na^+-P_i transport system in isolated myocytes of rat heart and in sealed sarcolemmal vesicles of bovine heart (184). Furthermore, this ROS-induced inhibition was reversed with catalase and SOD. The effect of ROS on Na^+-HCO^-_3 and K^+-HCO^-_3 cotransporters is unknown.

THE PRIMARY TRANSPORT PATHWAY AS A TARGET FOR ROS

ROS-induced changes in membrane properties are considered early events in response to oxidative stress. However, the molecular mechanism(s) for ROS action on ion transport pathways is not known. Hypothetically, the effects of ROS can be caused via direct effects on ion transport proteins. Ion channels

that have been thought to be a prime ROS target include a 106-kDa Ca^{2+}-release channel (162, 185), DHPR and RyR Ca^{2+}-release channels (125), and K^+ channels (94, 104). It has been reported that the direct effect of H_2O_2 on K_{ATP} channels in skeletal muscle is mediated via oxidation of the channel protein (179). It has to be noted that the concentration of H_2O_2 used by Weik and Neumcke (179) greatly exceeded those reported in studies where the effect was thought to be indirect (50, 123). Tokube et al. (169) suggested that ROS directly affected the K_{ATP} channel by binding to the ATP-binding site, causing a decrease in the sensitivity of the channel to ATP in the range of 0.2-2 mM, without affecting ADP or glibenclamide binding sites. Indirect effects of ROS on ion transport pathways are mediated via membrane phospholipids. There are several examples where changes in ion transport have been attributed primarily to changes in membrane phospholipids. It has been argued that ROS caused peroxidation of membrane phospholipids and that this led to changes in the K_{ATP} channel (63) and the Ca^{2+}-Mg^{2+}-ATPase (see Table 7).

The data in Tables 1-9 show that the concentrations of ROS-induced changes are different for ion channels, pumps, and exchangers. It appears that the inhibitory concentrations for ion pumps are less than those required for ion channel inhibition. Thus ion pumps are more sensitive to ROS than ion channels. However, it is not known which of the ion pumps is the primary target. Attempts have been made to determine the primary ion pathway that is affected by ROS from the IC_{50} of individual ROS. The data in Tables 7 and 8 show that Ca^{2+} uptake is more sensitive to H_2O_2 than ouabain-sensitive Rb^+ uptake. However, Rb^+ uptake is more sensitive to O^-_2 than Ca^{2+} uptake (42). These findings suggest that the primary ion pump that is affected by ROS depends not only on the type of pump but also on the individual ROS.

MECHANISMS OF ROS-INDUCED MODIFICATIONS

Figure 3 shows the possible molecular targets underlying ROS effects on ion transport mechanisms. These molecular targets include *1*) membrane phospholipids, *2*) membrane proteins, *3*) regulators of ion transport mechanisms, or *4*) a combination of these targets.

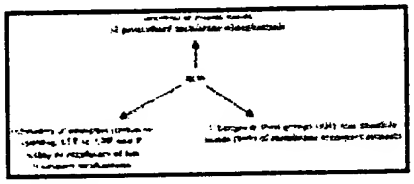

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Fig. 3. Molecular targets underlying ROS-induced malfunction of transport mechanisms.

Oxidation of SH Groups

The majority of studies of ROS effects on ion transport assume that ROS-induced stimulation (e.g., Ref. 12) or inhibition (e.g., Ref. 18) are mediated via modifications in SH groups of the transport proteins (see Tables 1-9). The interaction of ROS with ion transport proteins is viewed as being consistent with a thiol-disulfide redox state model and thus explains the widespread ROS-induced cellular dysfunction (85). The evidence for SH groups of the ion transport pathways as the site(s) for ROS action is discussed in *ROS mimic SH-oxidizing agents* and *SH-reducing agents reverse ROS action.*

ROS characteristics. ROS are capable of reaching SH groups embedded in the membrane. For example, H_2O_2 can readily cross the cell membrane and be converted to · OH via the Fenton reaction, with consequent oxidizing of the SH groups. This SH oxidation produces intermolecular cross-links that underlie ROS-induced protein oligomers (70, 80, 88). The physical changes in the structure of the channel and pump proteins modify the function of the transporting proteins and/or the availability of regulatory sites on these proteins. It has been proposed that H_2O_2 modifies the redox state of the channel protein in such a way that oxidation of the cysteines involved in the "ball" and "chain" mechanism that gate the channel occurs (173). Jamme et al. (80) suggested that, during exposure to ROS-generating systems, the Na^+-K^+-ATPase forms cross-links without the isoforms of the 90-kDa α-catalytic subunit and thus modifies the affinity and accessibility to the regulatory sites on this pump. Physical changes that modify ion transport mechanisms could also be brought about via ROS-induced changes in the properties of the phospholipids. The affinity or the accessibility of the ATP and ouabain binding sites could be modified by alteration in membrane integrity and fluidity during ROS-induced lipid peroxidation.

ROS mimic SH-oxidizing agents. It has been found that the SH-oxidizing agents H_2O_2 or DTNB prevent Ag^+ contractions and Ag^+ inhibition of E-C coupling in single skeletal muscle fibers from *R. temporaria* or *R. catesbeiana* and that these effects were reversible with the SH-reducing agents (125). The RyR Ca^{2+}-release channels can also be enhanced by ROS and SH-oxidizing agents that induce Ca^{2+} release (Table 1 and Refs. 76, 145, 170, 188).

The oxidation state of ion transport proteins does not simply favor an active state, and the reduced state does not favor an inactive state. For example, oxidization of SH groups by DTNB reversibly increased the activity of maxi-K_{Ca} channels in rabbit pulmonary and ear arterial smooth muscle cells (128), whereas oxidation induced inhibition of this channel that could be activated with GSH in equine tracheal myocyte (178). SH oxidation with DTNB and thimerosal also inhibited K_{Ca} channels (18) and the ATP-regulated K^+ channel in pancreatic β cell (75). Similarly, SH-oxidizing agents Hg^{2+} and thimerosal induced rapid and reversible block, with DTT, of the single Ca^{2+}-activated nonselective cation channel activity from brown fat cells (92). Internal oxidative agents used on ion channels show that DTNB and *p*-chloromercuriphenylsulfonic acid inhibited the activity of the K_{ATP} channel (25), and DTDP (lipophilic SH-oxidizing agent) and thimerosal (hydrophilic SH-oxidizing agent) inhibited the activity of the cloned rabbit smooth muscle L-type Ca^{2+} channels (23). All these findings indicate that substances that modify the SH groups also affect the activity of transport protein.

ROS that mimic the action of SH-oxidizing agents may act via a different mechanism. It is suggested that oxidation of K_{Ca} channels by H_2O_2 forms disulfide bonds that differ from those induced by SH oxidation with DTNB and thimerosal (18). There are several examples to support this suggestion. DTDP increased the P_o of the ATP-sensitive SCl channel (95, 96) and also activated H_2O_2-induced inhibition of this channel (unpublished observations). Similarly, the inhibitory effects of DTNB on whole cell Ca^{2+} and K^+ currents in β cells and the effectiveness of H_2O_2 suggest that these known SH-oxidizing agents act differentially (100, 101). It is also possible that oxidizing agents, e.g., oxidized glutathione (GSSH) and DTNB, could have different effects on the same channel (23).

SH-reducing agents reverse ROS action. ROS-induced changes that have been reported to be reversed with SH-reducing agents, e.g., DTT, include *1*) H_2O_2-induced increase in P_o of RyR in both cardiac and skeletal muscle (46, 124), *2*) H_2O_2-induced decline in activity of Ca^{2+}-activated K^+ channels (18), *3*) H_2O_2-induced depression in the Ca^{2+} pump (86, 87), *4*) UV-C-generated · OH and peroxyl (ROO ·)- or H_2O_2-induced inhibition of Na^+-K^+-ATPase (80, 85), and *5*) H_2O_2-induced inhibition of the Na^+/Ca^{2+} exchanger (88). Cysteine block of ROS-induced inhibition of the SR Ca^{2+} pump also suggests the involvement of SH groups (164). In addition, ROS-induced mechanical dysfunction, due to impairment of Ca^{2+}-ATPase, is prevented by SH-reducing DTT (38, 39, 46). It is assumed that SH-modifying agents act on ROS-induced disulfide by dissociating the H_2O_2-induced disulfide-linked RyR protein complex (45). However, the possibility that DTT has its own effect cannot be ruled out. Cai and Sauvé (18) suggested that oxidation of K_{Ca} channels by H_2O_2 forms disulfide bonds that differ from those induced by SH oxidation with DTNB and thimerosal.

Localization of the SH groups for ROS action. Localization of the SH groups on which ROS action occurs is achieved by using SH-modifying agents that differ in their pharmacological properties. The studies in which the poorly membrane-permeable thimerosal and the charged DTNB oxidizing agents were used suggest that H_2O_2 inhibits K_{Ca} channels by interacting with SH groups that are localized on the cytoplasmic side of the channel (see Ref. 18). On the other hand, rose bengal, a ROS-generating system that reverses the blocking effect of ryanodine (see Ref. 185), has an action that suggests a competition between ROS and ryanodine on a binding site that contains some SH groups. *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide labeling of cysteine indicates that this binding site (its oxidized SH groups keep the RyR in the active state) is embedded in the membrane away from the cytoplasmic side of the membrane (124). It is not known whether such differences in the localization of SH groups, cytoplasmic vs. internal, may account for differences in the proposed mechanisms of ROS action. The differences in the sensitivity of ion transport pathways to ROS-generating systems may be due to preferential binding of ROS to the SH groups of amino acids in the transport proteins (61). There is also evidence that indicates the presence of different sites underlying ROS-induced modifications in ion transport pathways. The opposite effects of H_2O_2 (inhibition) and DTDP (activation) on the gating of the SCl channel suggest that these oxidizing agents have different binding sites on the channel protein.

Krippeit-Drews et al. (100, 101) reported that DTNB inhibited both Ca^{2+} and K_{ATP} currents, whereas H_2O_2 had no effect on the Ca^{2+} current while it enhanced the K_{ATP} current. These data point to the presence of another mechanism, other than SH oxidization, that may also be responsible for modulating ion channels. The presence of such different mechanisms may explain the opposite effects of H_2O_2 (12) and of $^1O_2$ and O^-_2 radicals (69) observed on the RyR Ca^{2+}-release channel. There is also evidence that the SH group modulating ATP-sensitive channels may be close to the ATP binding site. ATP inhibition of the K^+ channel prevents the irreversible inhibitor NEM from reaching critical SH groups (179). Similarly, ATP inhibition of the SCl channel prevents the oxidizing agent DTDP from activating the channel (unpublished observations).

Other SH-modulated transport proteins. Some of the ion channels that are modulated by SH reagents have also been modulated by ROS in accordance with the SH hypothesis. One would expect that all ion channels and pumps that are modulated by SH-reducing and SH-oxidizing agents would also be modulated by ROS and the oxidation-reduction state in vivo. However, it should not be assumed that ROS would act in a manner similar to SH-oxidizing agents. As indicated above, there is evidence, contrary to such similarities, pointing to different mechanisms of actions. Some of the ion channels that are modulated by SH reagents, and not yet examined for ROS effects, include fast transient K^+ ($I_{K(A)}$) channels (141), diphtheria toxin channels (116), and reduced human skeletal macroscopic Cl^- current (hClC-1) (105).

Changes in Ca^{2+} Homeostasis

Intracellular Ca^{2+} is an important second messenger system, and various cells maintain Ca^{2+} homeostasis. ROS-induced functional abnormalities in cardiac muscle are thought to be linked to an increase in $[Ca^{2+}]_{cyt}$ (see Refs. 49 and 51), which has been confirmed with the fura 2 technique (16, 63). The broad effects of ROS can also be explained in terms of changes in the Ca^{2+} second messenger system. In cardiac tissue, the elevation of cytosolic Ca^{2+} (Ca^{2+} overload) is linked to various functional abnormalities, e.g., contractile dysfunction and ventricular arrhythmia, associated with ROS-induced tissue damage during ischemiareperfusion (51). ROS-induced changes in $[Ca^{2+}]_{cyt}$ homeostasis of muscles in general could be mediated via depression in sarcolemmal Ca^{2+}-ATPase, inhibition in SR Ca^{2+}-ATPase (Table 7), modification in the gating of SR Ca^{2+}-release channels (Table 2), changes in the Na^+/Ca^{2+} exchanger (Table 9), or nonspecific Ca^{2+} leakage across membranes (see Ref. 161). The changes in Ca^{2+} homeostasis need not be directly due to ROS-induced modifications in Ca^{2+} pathways but may also arise indirectly via modifications in other ion pathways. Cai and Sauvé (18) have argued that H_2O_2 may modulate agonist-induced Ca^{2+} influx, activating nitric oxide synthase, which metabolizes L-arginine to citrulline and nitric oxide, indirectly via depolarization in the membrane potential due to H_2O_2-induced inactivation of K_{Ca} channels. The role of HOCl in increasing intracellular Ca^{2+} homeostasis (46) is partly due to its effects on both the sarcolemmal Na^+/Ca^{2+} exchanger (88) and the Na^+-K^+-ATPase (103, 114). In fact, some Ca^{2+} pathways are ruled out as a cause for changes in Ca^{2+} homeostasis. For example, the

irreversible free-radical-induced decrease in Ca^{2+} currents in ventricular myocytes suggests that cellular Ca^{2+} overload during reperfusion is unlikely to be due to an increase in the sarcolemmal Ca^{2+} influx via voltage-gated Ca^{2+} channels (48). Regarding the contribution of other Ca^{2+} pathways to changes in Ca^{2+} homeostasis, Elmoselhi et al. (43) found that the Ca^{2+} pump contributing to the IP_3-sensitive pool was damaged by H_2O_2 and O^-_2, whereas the Ca^{2+} pump contributing to the IP_3-insensitive pool was only damaged by H_2O_2. The IP_3-sensitive Ca^{2+} channel and a suspected RyR Ca^{2+}-release channel are less sensitive than the Ca^{2+} pump. Oxidant-induced changes in Ca^{2+} homeostasis are also reported to occur in neurons. For example, oxidation enhanced the aggregation of amyloid β protein (36) that forms Ca^{2+} channels (3), thus altering Ca^{2+} homeostasis to produce neurotoxicity (see Ref. 53).

Lipid Peroxidation

In addition to the direct effects of ROS on ion channels and pumps underlying the transmembrane signaling mechanism (see Ref. 181), ROS alter compartmentation and ionic homeostasis, via membrane phospholipids, leading to alteration in membrane function (16). It is important to distinguish between two possible consequences of ROS-induced lipid peroxidation. The first possibility is that ROS-induced lipid peroxidation leads to a nonspecific leak of some pathway in the lipid itself, which consequently results in a modification of Ca^{2+} homeostasis. The second possibility is that ROS-induced lipid peroxidation modifies the physical properties of phospholipids in such a way that some proteins of ion channels, pumps, exchangers, and/or associated proteins that regulate these transport pathways are altered. The first possibility can be ruled out, since there is overwhelming evidence suggesting that ROS induce specific effects on ion transport pathways (see Tables 1-9). The second possibility cannot be ruled out, as it does not exclude the possibility of direct ROS effects on proteins of the ion transport pathways. There is evidence for ROS-induced membrane peroxidation that causes membrane malfunction. Guerra et al. (58) found that anti-lipoperoxidant partially prevented DHF-induced reduction in the DHP binding sites and that the protectant thiourea (an · OH scavenger) prevented lipoperoxidative damage (80). It has also been demonstrated that the Na^+/Ca^{2+} exchanger is sensitive to lipid composition (10) and is enhanced by increasing cholesterol content (106). Similarly, the Na^+-K^+-ATPase is activated by fatty acids, acylglycerols, and related amphiphiles (79). It has also been reported that membrane lipid peroxidation by *t*-BHP modified the physiological automaticity by impairing cellular metabolic functions and damaging lipid membrane structure and ion channel proteins (147).

The mechanism of ROS-induced membrane peroxidation involves biochemical changes that alter the physical properties and inactivate membrane-bound enzymes that regulate membrane permeability. Indeed, loss of endothelial cells, which are a major source of reperfusion-generated free radicals, has been found to be associated with increased formation of lipid peroxidation products, such as malondialdehyde and lipid peroxides (see Ref. 98). There is evidence that lipid peroxidation subsequently leads to alterations in Ca^{2+} homeostasis (see Ref. 63). For example, *t*-BHP augments and subsequently attenuates Ca^{2+} currents in rabbit sinoatrial node and nodal isolated cells. Modification by *t*-BHP of Ca^{2+} homeostasis has also been deduced from an increase in resting tension (122). Therefore, lipid peroxidation has been invoked as a mechanism underlying some diseases. For example, Butterfield et al.

(17) reported that β-amyloid peptide free radical fragments initiated synaptosomal lipoperoxidation that has been implicated in Alzheimer's disease.

Oxidative Phosphorylation and ATP Levels

In endothelial cells there is evidence showing that ATP levels decline under conditions of oxidative stress or H_2O_2-induced inhibition of glucose-dependent pathways of ATP synthesis (68). Obviously, ATP-sensitive, e.g., K_{ATP} channels, or ATP-modulated transport pathways, e.g., Ca^{2+} and Na^+ pumps, are likely to be modified if the ATP levels are significantly reduced either *1*) directly via ROS-induced effects on the metabolism of ATP production or *2*) indirectly via ROS-induced splitting of the ATP to ADP and phosphate (see Ref. 169). It has been reported that H_2O_2 inhibits the glycolytic pathway and oxidative phosphorylation (72), causing an increase in the activity of the K_{ATP} channel (50, 123). However, the relationship between a decrease in ATP levels and cellular dysfunction is not clear (148). There is evidence showing that cytotoxicity is not coupled to ATP levels. For example, desferrioxamine, an iron chelator, and allopurinol and oxypurinol (XO inhibitors) prevent H_2O_2 cytotoxicity but not a decrease in ATP levels in pulmonary endothelial cells (172). Similarly, after ischemia the ATP level recovers on reperfusion, whereas Na^+-K^+-ATPase and the glycoside binding sites continue to decrease (see Refs. 82 and 90).

At the ion channel level, the X/XO- and H_2O_2-induced increase in the P_o of K_{ATP} channels recorded in the cell-attached configuration results from a reduction in ATP level due to irreversible inhibition of oxidative phosphorylation and glycolysis rather than to a reduction in the channel sensitivity to ATP (51). However, observations similar to those found in the cell-attached configuration (51) and in the inside-out configuration (32) have been attributed to a direct effect on the ATP sensitivity of the channel, thus ruling out inhibition of oxidative phosphorylation and glycolysis (169). These differences are thought to be due to *1*) differences in Mg^{2+} concentration levels, which affect K_{ATP} channels (169), and *2*) differences in ROS-generating systems, i.e., X/XO producing O^-_2 (169) and $H_2O_2/FeCl_2$ producing · OH (32).

Changes in pH

It is known that oxidant stress can modify some pH regulatory mechanisms (see Ref. 14). Subsequently, this causes changes in intracellular pH, which can influence various ion transport mechanisms, such as inactivation of enzymes, damage to Na^+-K^+-ATPase (90), and modification of Ca^{2+}-release channels (110, 139) and sarcolemmal Cl^- conductance (142). ROS-induced SR disruption in ischemic myocardium via interaction with H^+ has also been recognized (65). Initially, it was reported that O^-_2 acts as a signal for the increase in intracellular pH (155). Ikebuchi et al. (74) confirmed that HX/XO generating O^-_2 induced an immediate increase in the intracellular pH of human cultured amnion cells. This O^-_2-induced increase is not mediated via the Na^+/H^+ exchanger as indicated from the ineffectiveness of removing extracellular Na^+ or blocking its pathway with amiloride. Recently, Wu et al. (182) proposed that the

effects of H_2O_2 on cultured rat cardiac myoblasts are not mediated through a rise in intracellular Ca^{2+} or inhibition of oxidative phosphorylation. They proposed that ROS effects are mediated via induction of intracellular acidification. The mechanism by which ROS induce pH changes is via inhibition of glycolysis and hydrolysis of ATP rather than inhibition of Na^+/H^+ and Cl^-/HCO^- exchangers or a Na^+-HCO^-_3 cotransporter (182). These authors have shown that, in the cardiac cell model cell line, H9c2, the intracellular production of · OH and not O^-_2 or H_2O_2 is the cause of the acidification. Because it is known that acidosis modifies Ca^{2+}-release channels (110, 139) and reduces contraction in cardiac and skeletal muscles (44), Wu et al. (182) argued that the small · OH-induced acidification (22) in part contributes to the cardiac stunning seen during reperfusion-ischemia by means of either decreasing the sensitivity of the contractile elements to Ca^{2+} concentration or reducing Ca^{2+} release from the SR. It should be noted that, according to the pH hypothesis, the effects of ROS on ion transport pathways are indirectly mediated via changes in pH. However, single ion channel studies that show the effects of H_2O_2 on the RyR Ca^{2+}-release channel in artificial bilayer experiments where the pH is constant indicate that H_2O_2 directly affects the RyR Ca^{2+}-release channel (12).

ROS AS SECOND MESSENGERS IN ION TRANSPORT PATHWAYS

Recent reports suggest that ROS, or at least H_2O_2, may function as second messenger systems. It has been proposed that H_2O_2 modulates a complex of heme-linked NADPH oxidase protein coupled to K^+ channels that function as an oxygen sensor mechanism in airway chemoreceptors of small lung carcinoma cell lines (176). Closure of this K^+ channel induces membrane depolarization and enhances Ca^{2+} influx that could cause the release of transmitters or modification of spike duration and frequency (176). It is assumed that, for H_2O_2 to play a second messenger role, a specificity to H_2O_2 modulation must be achieved, as well as sufficient concentrations of H_2O_2 accumulated, before it is destroyed by H_2O_2 scavengers in a highly reduced cellular environment, e.g., the presence of 1 mM GSH (see Refs. 83, 173, 189). Another channel that is modulated by ROS in a second messenger manner is the SCl channel. Pharmacological and biophysical studies indicate the presence of an O_2-sensing mechanism (GSH-GSSH) on the SCl channel protein (95) that is also modulated by H_2O_2 (unpublished observations). It remains to be seen whether the O_2-sensitive K^+ channel of the arterial chemoreceptor that is modified by low PO_2 (47) is sensitive to ROS. It has been reported that an anion channel allows · O^-_2 permeation into human amnion cells, which consequently causes increases in *1*) cytosolic pH, *2*) $[Ca^{2+}]_{cyt}$, and *3*) release of arachidonate (74). The interaction of ROS with other second messenger systems could also lead to changes in Ca^{2+} levels, e.g., H_2O_2-induced activation of phospholipase A_2 and arachidonic acid metabolic pathways (21, 22).

CONCLUSIONS

Top
Abstract
Introduction
Conclusions
References

The well-accepted ROS-induced cardiac dysfunction during ischemia and perfusion, cardiomyopathies, neurotoxicity, inflammation, and aging involves the disruption of various ion transport pathways underlying electrophysiological functions. ROS modify ion transport mechanisms either directly via ion transport pathway proteins and/or ion transport regulatory proteins or indirectly via peroxidation of membrane phospholipids. The nature and sequence of events that lead to the disruptions of these ion transport pathways are not fully understood. ROS-induced modification of SH groups on ion transport proteins leads to changes in the homeostasis of Ca^{2+}, a major second messenger system, and perhaps other cytosolic factors. The order of potency and the primary mechanism of cell dysfunction for individual ROS are yet to be determined. The potency of individual ROS and the ion transport mechanism that they primarily affect depend on various factors that include types of tissue. It is obvious, therefore, that such understanding is important for the development of specific drugs for individual ion transport proteins. ROS scavengers, e.g., superoxide dismutase and catalase, thiol-disulfide modifying agents, and Ca^{2+} channel modulators, are the bases for therapeutic approaches in free radical-induced ischemic and reperfusion myocardial injury. The cloning of ion transport protein isoforms, utilization of specific antibodies and molecular probes, and direct mutations of specific sites, will enable us to characterize the SH-oxidization sites and enhance our understanding of the structure-function relation for individual transport proteins.

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FOOTNOTES

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Top
Abstract
Introduction
Conclusi ns
References

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